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LncRNA HOTAIRM1 inhibits the progression of hepatocellular carcinoma by inhibiting the Wnt signaling pathway

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Abstract. – OBJECTIVE: To explore the possible role of long non-coding RNA (lncRNA) HOTAIRM1 in the pathogenesis of hepatocellular carcinoma (HCC) and its underlying mechanism.

PATIENTS AND METHODS: LncRNA HOTAIRM1 expressions in 30 pairs of hepatocellular carcinoma tissues and paracancerous tissues were detected by quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR). The survival analysis and receiver operating characteristic (ROC) curve were introduced to explore the relationship between lncRNA HOTATRM expressions and prognosis of HCC patients. The correlation between overall survival and clinical variables of HCC patients was estimated by single-factor and multiple-factor regression analysis, respectively. Overexpression plasmid of lncRNA HOTAIRM1 was designed and transfected into HCC cells according to the instructions of Lipofectamine 2000. Cell proliferation and apoptosis were detected by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively. Moreover, expressions of apoptosis-related genes and the Wnt pathway-related proteins were detected by Western blot.

RESULTS: Lower lncRNA HOTAIRM1 expressions were observed in the HCC tissues than those of the paracancerous tissues. ROC curve indicated a high sensitivity and specificity of lncRNA HOTAIRM1 for HCC. PFS in HCC patients was correlated with tumor size and lncRNA HOTAIRM1 expression, whereas not correlated with age, sex, GGT, AFP, Child-Pugh grade, HBsAg, cirrhosis, number of tumors, micro-vessel metastasis, tumor differentiation, and TNM stage of HCC. Overexpression of HOTAIRM1 led to decreased proliferative ability and increased apoptosis of HepG2 and HHCC cells. In addition, overexpressed lncRNA HOTAIRM1 remarkably increased the expression of apoptosis promotor Bax, but decreased the expressions of apoptosis inhibitors Bcl-2 and Bid. Meanwhile, expressions of related proteins in the Wnt pathway were decreased as well.

CONCLUSIONS: HOTAIRM1, which was downregulated in HCC, might inhibit the proliferative ability and promote the apoptosis of HCC cells by suppressing the Wnt pathway, thereby inhibiting the progression of hepatocellular carcinoma.

Key Words
Hepatocellular carcinoma, LncRNA HOTAIRM1, Wnt pathway, Proliferation, Apoptosis.

Introduction

Hepatocellular carcinoma (HCC) is a malignant tumor developed from hepatocytes. Epidemiological investigations[1-3] have indicated that HCC has become the third major cause of tumor death throughout the world and the second leading cause of tumor death in China. Due to the large number of patients with hepatitis B in China, about 383,000 people die from HCC every year, accounting for 50% of the deaths of HCC worldwide. With the progress of medical technology, there are various methods for the treatment of HCC, such as surgical resection, liver transplantation and chemotherapy, thus greatly improving the survival of HCC patients. However, HCC has a high metastasis rate when first diagnosed, resulting in a low survival of HCC. Therefore, explorations on HCC development and its molecular biomarkers have been well recognized. In recent years, several studies have found that long non-coding RNA (lncRNA) is correlated with the tumor development, which provides a new direction for the diagnosis and treatment of HCC.

With the completion of human genome and the development of the sequencing technology,

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the human transcriptome has been well identified and greatly promoted. In 2002, Okazaki et al[4] identified a large number of long non-coding RNA transcripts in the full-length cDNA library of mice, thus proposing the concept of lncRNA. With the deepening of relevant researches, especially the study of lncRNA HOTAIR in 2007, accumulating lncRNAs have been proved to play a regulatory role in various diseases, including malignant tumors[5].

LncRNA HOTAIRM1, has been proved to be related to the formation and differentiation of myeloid cells, which was initially found in the medullary cell system in 2009 by Zhang et al[6]. In addition, other studies have shown that lncRNA HOTAIRM1 is expressed in fetal brain tissues and may be correlated with neuronal differentiation[7]. It has been found that the lncRNA HOTAIRM1 expressions in colorectal cancer tissues are remarkably lower than those of paracancerous tissues. Moreover, plasma expressions of lncRNA HOTAIRM1 in colorectal cancer patients are lower than those of the control group. Meanwhile, lncRNA HOTAIRM1 knockdown promotes cell proliferation[8]. However, higher lncRNA HOTAIRM1 expressions in pancreatic ductal adenocarcinoma tissues are observed in comparison with paracancerous tissues[9]. So far, there are no researches on the relationship between lncRNA HOTAIRM1 and HCC. In the present study, we first detected the lncRNA HOTAIRM1 expression in HCC tissues. The effects of lncRNA HOTAIRM1 on the proliferative and apoptosis abilities of HCC cells were further analyzed, thus providing new ideas for treating HCC.

Patients and Methods

Collection and Preservation of HCC Specimens

Tumor tissues and the corresponding paracancerous tissues of HCC patients with surgical resection in our hospital from July 2002 to February 2007 were collected. Enrolled patients were pathologically diagnosed as HCC and did not receive any preoperative treatment. Briefly, tumor tissues of HCC patients were collected within 10 min in avoidance with the necrotic area of the tumor center. The corresponding paracancerous tissues away from the edge of tumors (> 2 cm) were collected. All collected specimens were immediately placed in cryopreservation tubes and stored in liquid nitrogen for tissue RNA and protein extraction. This study was approved by the Ethics Committee of Provincial Hospital Affiliated to Shandong University. Signed written informed consents were obtained from all participants before the study.

RNA Extraction

1 mg of collected tissue was ground to powder by adding liquid nitrogen. After tissues were lysed by 1 ml of TRIzol (Invitrogen, Carlsbad, CA, USA), 200 μL of chloroform was added for the following centrifugation at 12,000 g for 15 min. Isodose of pre-cooled isopropanol was added to the supernatant for another 15 min-centrifugation. Pre-cooled 75% ethanol was added after the supernatant was discarded. Finally, 10 μL of diethyl pyrocarbonate (DEPC) water was utilized for dissolving the obtained mRNA.

qRT-PCR (quantitative Reverse Transcriptase-Polymerase Chain Reaction)

The extracted mRNA was reversely transcribed to complementary Deoxyribose Nucleic Acid (cDNA) according to the instructions of First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The qRT-PCR reaction conditions were as follows: pre-denaturation at 95°C for 30 s, followed by denaturation at 95°C for 5 s, and annealing and extension at 60°C for 30 s, with a total of 40 cycles. Analysis of melting curves were 95°C for 5 s and 60°C for 1 min. Each sample was repeatedly performed for 3 times. Primers used in this study were: HOTAIRM1: F: 5'-TGGAG'TTGGGGGTTTCTGTA-3', R: 5'-TTCAGTGCACAGGTTCAAGC-3', GAPDH: F: CGCTCTCTGCTCCTCCTGTTC, R: ATCCGTTGACTCCGACCTTCAC.

Cell Culture and Transfection

The normal liver cell line (L-02) and the HCC cell lines (HepG2, HB611, and HHCC) were purchased from ATCC (Manassas, VA, USA). Cells were cultured in completed high glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), and were maintained in a 5% CO_2 incubator at 37°C. Cells were seeded into the 6-well plates when the cell confluence was up to 80-95%. Cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were collected and seeded into the 96-well plates. After 24 h-inoculation, 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added into each well and incubated for 1 h in dark. The absorbance (OD) values at the wavelength of 450 nm were accessed with a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell Apoptosis

Transfected cells were digested with trypsin and centrifuged at 1000 r/min for 3 min. Cell suspension was prepared by adding 1 mL of phosphate-buffered saline (PBS) and then centrifuged at 1500 r/min for another 3 min. Cells were incubated with antibodies at room temperature for 15 min in the dark. Subsequently, the apoptosis rate was analyzed by flow cytometry.

Western Blotting

The total protein of transfected cells was extracted. The concentration of each protein sample was determined by a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). Briefly, 50 μg of total protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Membranes were blocked with 5% skimmed milk, followed by the incubation of specific primary antibodies overnight. Membranes were then incubated with the secondary antibody at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Statistical Analysis

We used statistical product and service solutions (SPSS19.0, IBM Corp., Released 2011. IBM SPSS Statistics for Windows, Armonk, NY, USA) software for all statistical analyses. The quantitative data were represented as $\bar{x}\pm s$. The *t*-test was used for comparing differences between the two groups. Survival analysis and ROC curve were introduced by Graphpad (La Jolla, CA, USA). The χ^2-test was used to analyze the correlation between clinical characteristics of HCC patients and the lncRNA HOTAIRM1 expression. Single-factor and multiple-factor regression analysis were performed to investigate the correlation between PFS and clinical variables of HCC patients. $p<0.05$ was considered statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

LncRNA HOTAIRM1 Was Down-Regulated in HCC

We detected the lncRNA HOTAIRM1 expressions in 30 pairs of HCC and paracancerous tissues by qRT-PCR. Lower expressions of lncRNA HOTAIRM1 were observed in HCC tissues than those of the paracancerous tissues ($p<0.001$, Figure 1A, 1B). We found that the area under the ROC curve was 0.7533 and *p* was 0.0007550, indicating that the lncRNA HOTAIRM1 expression was highly sensitive and specific for HCC (Figure 1C). Subsequently, HCC patients were assigned into the high expression group and low expression group based on the lncRNA HOTAIRM1 expression. Notably, our finding demonstrated that the survival time of HCC patients with low lncRNA HOTAIRM1 expression was significantly reduced compared with those of high expression group ($p=0.0299$, Figure 1D). The survival of HCC patients was correlated with tumor size and lncRNA HOTAIRM1 expression, whereas not correlated with age, sex, GGT, AFP, Child-Pugh grade, HBsAg, cirrhosis, number of tumors, micro-vessel metastasis, tumor differentiation, and TNM stage (Table I). These results all suggested that lncRNA HOTAIRM1 might be involved in the HCC development and could be served as an indicator of clinical diagnosis and prognosis of HCC.

LncRNA HOTAIRM1 Inhibited Proliferative Ability and Promoted Apoptosis of HCC Cells

To investigate the effect of lncRNA HOTAIRM1 on HCC cells, mRNA expressions of lncRNA HOTAIRM1 in the HCC cell lines (HepG2, HB611, and HHCC) and the normal liver cell (L-02) were detected by qRT-PCR. LncRNA HOTAIRM1 was down-regulated in HCC cells in comparison with that of normal liver cells (Figure 2A). Therefore, HepG2 and HHCC cells were selected for subsequent overexpression experiments. Transfection efficacy of lncRNA HOTAIRM1 overexpression plasmid was shown in Figure 2B and 2C. Subsequently, the proliferative ability of HepG2 and HHCC cells were detected by CCK-8 assay after transfection for 6 h, 24 h, 48 h, and 72 h, respectively. The results indicated that cell viability of HCC cells was significantly reduced after lncRNA HOTAIRM1 overexpression (Figure 2D, 2E). Furthermore, KEGG pathway enrichment analysis suggested that the function of lncRNA HOTAIRM1 was mainly enriched in the apoptotic pathway (Figure 2F). Therefore, we evaluated

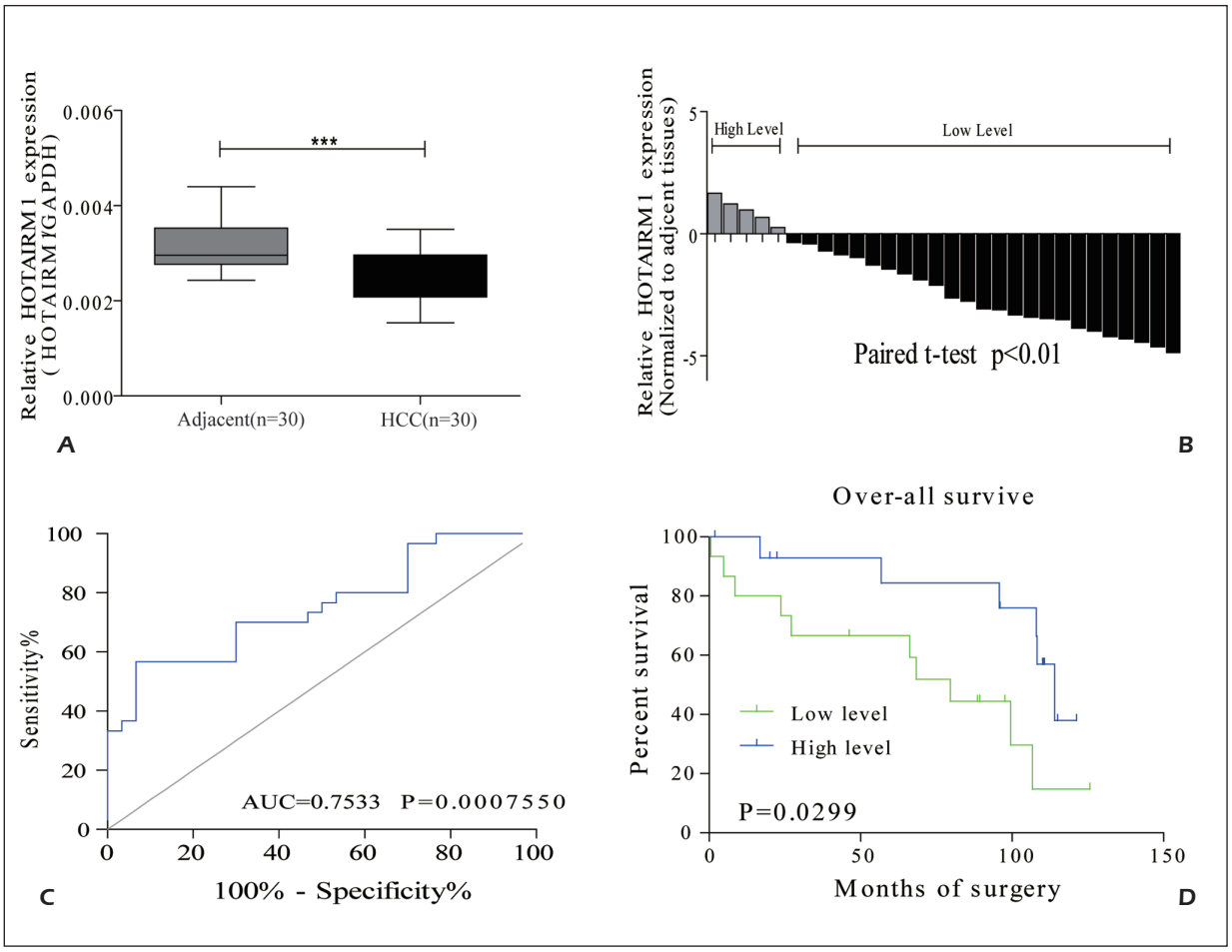

Figure 1. LncRNA HOTAIRM1 was down-regulated in HCC. -**B**, The expressions of lncRNA HOTAIRM1 in HCC tissues were significantly lower than those of the paracancerous tissues ($p<0.001$). **C**, The ROC curve of lncRNA HOTAIRM1 in HCC and paracancerous tissues (AUC=0.7533, $p=0.0007550$). **D**, The survival time of HCC patients with high lncRNA HOTAIRM1 expression was significantly longer than those with low expression ($p=0.0299$).

Table I. Factors for Cox regression analysis.

Variable name	Univariate analysis		Multivariate analysis	
	p-value	HR	*p*-value	HR
Age (≤50 *vs.* >50)	0.7425	1.2199	0.2691	1.1021
Sex (female vs. male)	0.9515	0.9933	0.3720	1.2011
GGT, U/l (≤54 *vs.* >54)	0.3125	0.9956	0.4561	0.6549
AFP, ng/mL (≤20 *vs.* > 20)	0.2453	1.1132	0.2087	1.5082
Child-Pugh grade	0.1716	1.0934	0.7742	0.9592
HBsAg (negative *vs.* positive)	0.3520	1.3365	0.5046	1.2090
Liver cirrhosis (no *vs.* yes)	0.7194	1.7259	0.2664	1.4533
Tumor number (single *vs.* multiple)	0.8022	0.6237	0.0894	0.6871
Tumor size, cm (≤5 *vs.* > 5)	0.0124	1.5274	0.0423	1.6261
Microvascular invasion (no *vs.* yes)	0.6721	2.5515	0.7892	1.2308
Tumor differentiation (I + II *vs.* III + IV)	0.3734	1.7949	0.5971	1.9951
TNM stage (I *vs.* II + III)	0.7042	1.1907	0.3345	0.9865
HOTAIRM1 (low *vs.* high)	0.0198	0.7974	0.0436	0.5543

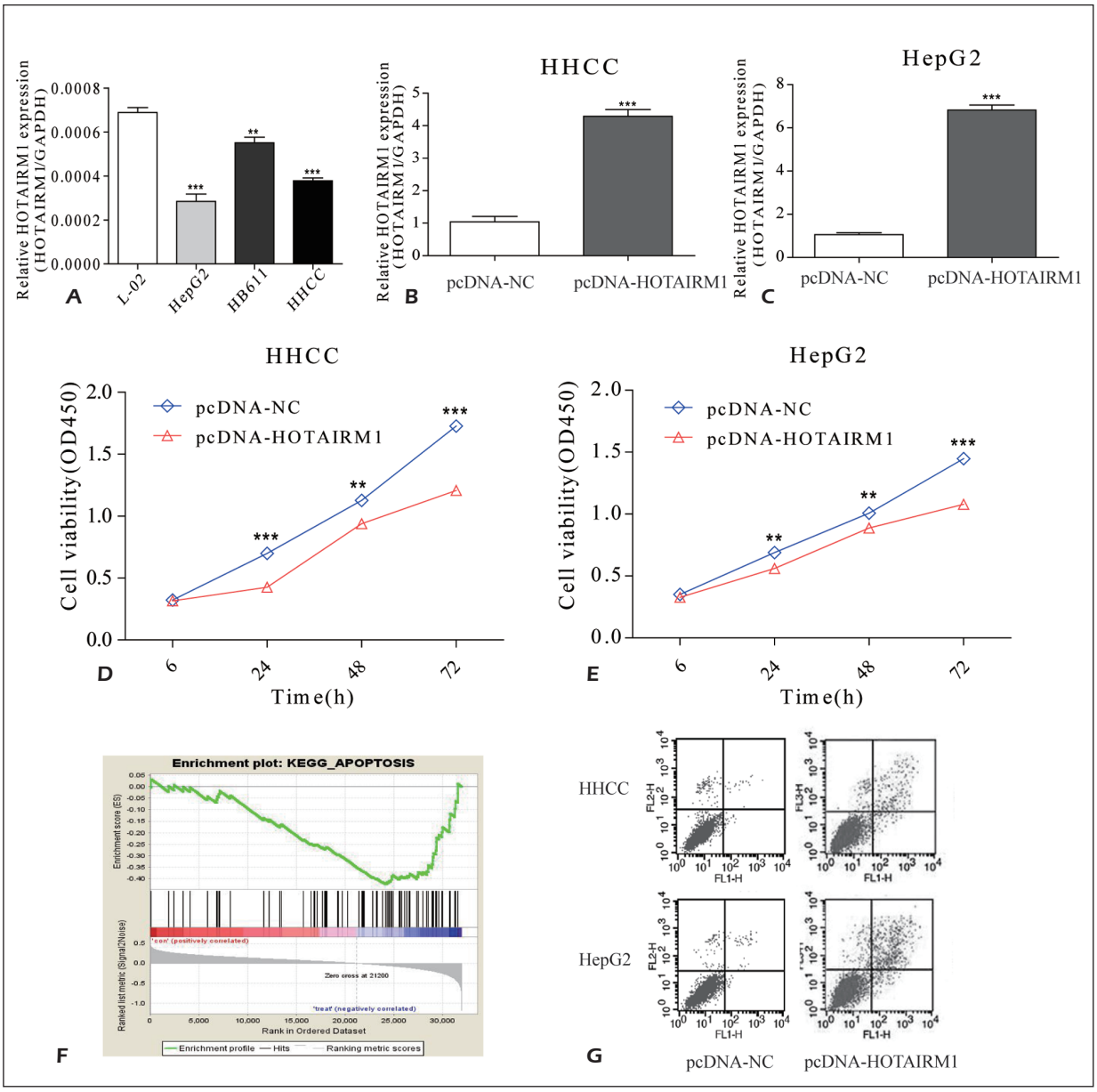

Figure 2. LncRNA HOTAIRM1 inhibited proliferation and promoted apoptosis of HCC cells. **A**, The lncRNA HOTAIRM1 expression in the HCC cell lines (HepG2, HB611 and HHCC) and the normal liver cell line (L-02). **B**-**C**, The transfection efficacy of pcDNA-HOTAIRM1 in HepG2 and HHCC cells. **D**-**E**, After overexpression of lncRNA HOTAIRM1 in HepG2 and HHCC cells, cell viability was decreased detected by CCK-8 assay. **F**, KEGG pathway enrichment analysis showed that the function of lncRNA HOTAIRM1 was mainly enriched in the apoptotic pathway. **G**, Cell apoptosis was increased after overexpression of lncRNA HOTAIRM1 in HepG2 and HHCC cells.

the apoptosis of HCC cells after lncRNA HOTAIRM1 overexpression. Our data demonstrated that apoptosis rate was remarkably increased in HepG2 and HHCC cells after overexpression of lncRNA HOTAIRM1 (Figure 2G). All these findings suggested that lncRNA HOTAIRM1 inhibits its proliferative ability and promotes apoptosis in HCC cells.

Mechanism of lncRNA HOTAIRM1 in inhibiting the HCC progression

Next, we explored how lncRNA HOTAIRM1 inhibited the HCC progression. Overexpressed lncRNA HOTAIRM1 in HepG2 and HHCC cells increased the expression of apoptosis promotor Bax, while decreased the expressions of apoptosis inhibitors Bcl-2 and Bid (Figure 3A-

C). These results further indicated that lncRNA HOTAIRM1 could enhance the apoptosis rate of HCC cells. It is well recognized that the Wnt pathway is involved in the development and progression of malignant tumors by regulating cell apoptosis. Therefore, we detected expressions of Wnt pathway-related proteins, including Akt1, pGSK-3β, and β-catenin. Surprisingly, we found that expressions of the related proteins were significantly decreased after lncRNA HOTAIRM1 overexpression (Figure 3D-F), indicating that the Wnt pathway was suppressed. The above results illustrated that lncRNA HOTAIRM1 might promote apoptosis of HCC cells by inhibiting the Wnt signaling pathway.

Discussion

HCC is one of the most common malignant tumors in China[9]. At present, surgical treatment is still the main method for the treatment of HCC. However, most of the HCC patients are already in the late stage when they are first diagnosed. So far, there are no other effective treatments for HCC, which leads to the low 5-year survival rate of HCC[11,12]. Currently, mechanisms of the HCC occurrence are still unclear[13]. Therefore, it is of great importance to explore the pathogenesis of HCC, thus providing new directions in searching for the effective HCC drugs.

LncRNA is a kind of non-coding RNA with over 200 nucleotides in length[14], the amount of which is far more than those of the known mRNAs and miRNAs[15]. Recent researches have demonstrated that lncRNA exerts a crucial role in various life activities, such as dosage compensation effect, epigenetic regulation, and post-transcriptional regulation[16]. Accumulating studies have suggested that lncRNA is of great importance in the development and progression of malignant tumors, which undoubtedly brings new directions for investigating the mechanism of tumor pathogenesis[16]. Therefore, systematic and in-depth in-

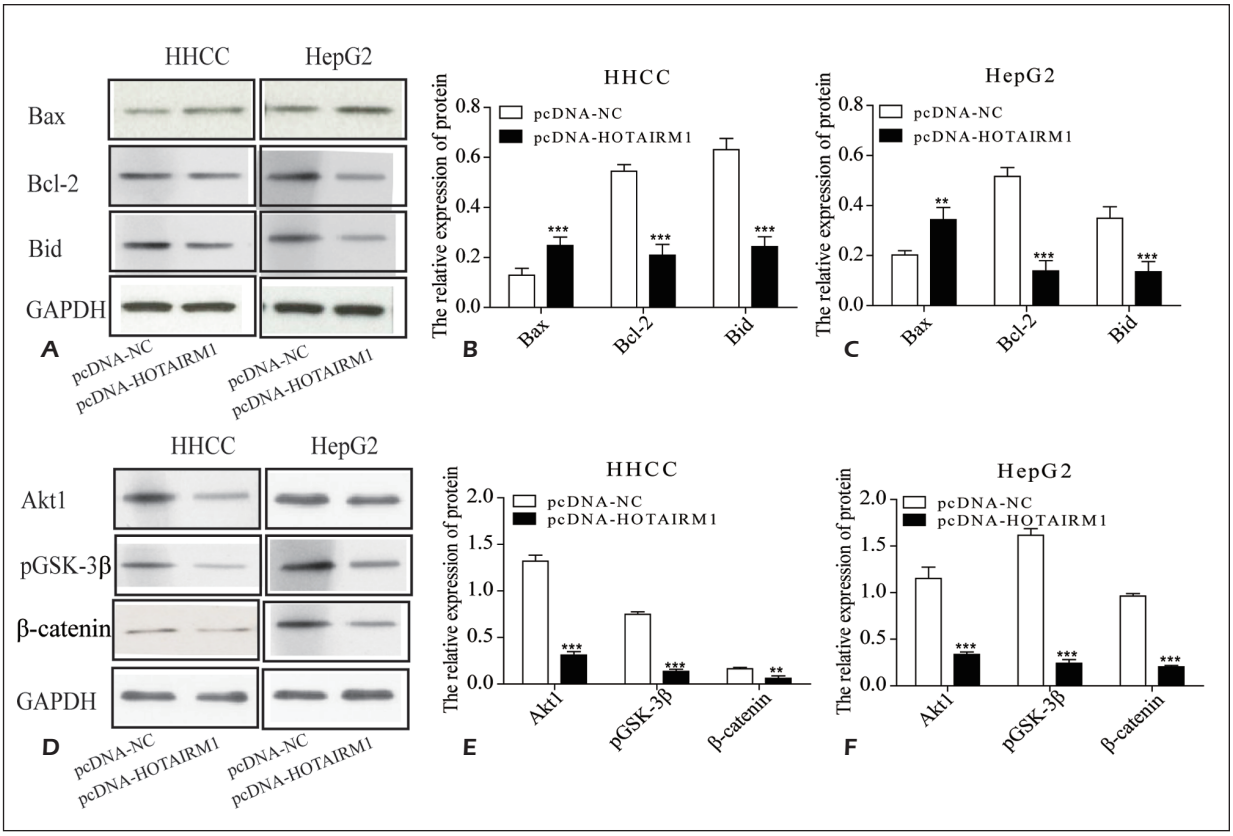

Figure 3. The mechanism of lncRNA HOTAIRM1 in inhibiting the progression of HCC. **A-C**, After overexpression of lncRNA HOTAIRM1 in HepG2 and HHCC cells, the expression of apoptosis promotor Bax was increased, while the expressions of apoptosis inhibitors Bcl-2 and Bid were decreased. **D-F**, After overexpression of lncRNA HOTAIRM1, related proteins (Akt1, pGSK-3β and β-catenin) of the Wnt pathway were significantly inhibited.

vestigations are urgently needed to improve the survival rate of HCC patients.

Multiple researches[18-23] have proved that lncRNAs can be served as molecular targets for predicting the prognosis of HCC patients, such as HULC, HOTAIR, and GASS. Tumor relapse is frequently seen in HCC patients with high expressions of HULC and HOTAIR, and low expression of GASS. These findings suggested that some certain lncRNAs may serve as prognostic biomarkers for HCC. LncRNA HOTAIRM1 is located in the gene cluster of HOX, a well-known gene family that is highly conserved in evolution. As greatly involved in the vertebrate growth and cell differentiation, HOX exerts an essential role in the development of the central nervous system, gastrointestinal tract, and limbs[24]. In this study, lncRNA HOTAIRM1 expression was significantly reduced in HCC tissues. Meanwhile, overexpressed lncRNA HOTAIRM1 could inhibit the proliferative ability of HCC cells.

Apoptosis is an autonomous and ordered regulatory of cell death[25]. Dysregulated tumor cells can escape from apoptosis and exhibit rapid proliferation, thus leading to unlimited growth[26]. Therefore, many researches have focused on the relationship between tumor occurrence and cell apoptosis. For example, some effective treatments are achieved by inducing apoptosis in HCC patients[27]. In the present study, overexpressed lncRNA HOTAIRM1 increased the expression of apoptosis promotor Bax, whereas decreased expressions of apoptosis inhibitors Bcl-2 and Bid.

The Wnt pathway is a classical pathway, which is closely associated with the occurrence, progression, and metastasis of many malignant tumors[28]. Interestingly, our findings pointed out that expressions of key genes related to the Wnt pathway were significantly reduced after lncRNA HOTAIRM1 overexpression, including Akt1, pGSK-3β, and β-catenin. Taken together, the effect of lncRNA HOTAIRM1 on HCC is regulated *via* the Wnt pathway.

Conclusions

We found that downregulated HOTAIRM1 in HCC might inhibit the proliferative ability and promote the apoptosis of HCC cells by suppressing the Wnt pathway, thereby inhibiting the progression of hepatocellular carcinoma.

Conflict of interest

The authors declared no conflict of interest.

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